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## **Final Technical Report**

For NASA Cooperative Agreement NCC 2-1111 Entitled:

**"THE MOLECULAR BASIS OF HYPERTHERMOPHILY:  
THE ROLE OF HSP60/CHAPERONINS IN VIVO"**

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*CASI*

The hyperthermophilic, acidophilic archaeon, *Sulfolobus shibatae*, grows between 55°C and 88°C at pH 2.0, (Grogan et. al., 1990). Like nearly all other organisms, its ability to survive at lethal temperatures is greatly enhanced by a brief exposure to a near-lethal temperature (heat shock); a response known as acquired thermotolerance. For *S. shibatae* acquired thermotolerance is observed if cells growing at normal temperatures (75°C) are heat shocked at 85-88°C and challenged at lethal temperatures of 90-95°C (Trent et. al., 1990; 1994). Unlike other organisms, which synthesize a variety of heat shock proteins (HSPs), *S. shibatae* synthesizes predominantly two 60 kDa HSPs. These HSPs, known as TF55 $\alpha$  and TF55 $\beta$  (Kagawa et al. 1995) are among the most abundant proteins in *S. shibatae* at normal growth temperatures and during heat shock they reach between 10% and 15% of total proteins. Their increased synthesis correlates with acquired thermotolerance and their role in this process is supported by experimental observations (Trent et. al., 1994).

TF55  $\alpha$  and  $\beta$  are isolated from cells associated with double-ring structures, each ring composed of nine protein subunits. This double-ring structure from *S. shibatae* has been referred to as a "chaperonin" and similar structures composed of related proteins in other archaea, have been referred to as "thermosomes" (Phipps et al., 1991). These archaeal double-rings are clearly structurally related to double-rings found in bacteria and eukarya known as chaperonins (Chen et al., 1994; Braig et al., 1994).

In this study, we aim to understand how *S. shibatae* copes with high temperatures. In particular, we investigated the role of the 60 kDa heat shock protein (HSP60 or chaperonin) with the hypothesis that chaperonin stabilizes the cell membrane under stressful conditions. To prove the hypothesis, this year two questions were addressed: (1) Is the chaperonin localized in the cytoplasm or on the cell membrane? (2) Does the chaperonin show affinity to lipid *in vivo*? In addition to those, we intensively studied newly discovered chaperonin-related protein,  $\gamma$ , to understand how it influenced the function of the other components of chaperonin and how their combined activities contributed to hyperthermophily.

## **I. Localization of The Chaperonin in The Cell**

To answer the question if the chaperonin is localized on the cell membrane to stabilize it, immunofluorescence microscopy was employed. The chaperonin in the cell was visualized by chaperonin antibody and fluorescein (FITC) labeled secondary antibody, and the image was compared with that of isocitrate dehydrogenase known as a cytosolic enzyme.

## **MATERIALS AND METHODS**

Immunofluorescence microscopy was done as described (Arigoni, et.al. 1995) with the following modifications. Fifty microliter of fixative (500  $\mu$ l of 16% paraformaldehyde and 1  $\mu$ l of 25% glutaraldehyde (Sigma, microscopy grade) dissolved in water) was directly added to 250  $\mu$ l of the culture of pH 2-3, which allowed the fixative to work only inside the cell under neutral pH. That prevented to crosslink the S-layer proteins on the outer surface, which should cause the cell shrunken. The fixed cell was placed on poly-L-lysine treated multi-well glass slide and washed with 100 mM Trizma base *S. shibatae* medium solution to stop the fixation and to permeabilize the cell. After the cell was washed with PBS, crossreactions with primary and secondary antibodies were performed for 1 hr each at room temperature. Rabbit polyclonal antiserum

raised against the chaperonin was purified with protein A column and used in the protein concentration of 38 µg/ml. FITC conjugated goat anti-rabbit antibody (multiple-labeling grade) was purchased from Jackson ImmunoResearch Laboratories and used in 1/200 dilution. Rabbit antiserum raised against isocitrate dehydrogenase (ICDH) from *Sulfolobus tokodaii* was a gift from Dr. Wakagi and used in 1/50 dilution.

## RESULTS AND DISCUSSION

In Fig. I-1, FITC signal from anti-ICDH antibody stays dominantly in the center region of the cell, which is indicating ICDH's localization in the cytoplasm. In contrast, the signal from anti-chaperonin shows peripheral pattern, which indicates the chaperonin is localized on the cell membrane. The same pattern was observed among the three cultures incubated at 60, 76, and 86°C. That suggests the chaperonin is localized on the cell membrane regardless of the cultivation temperatures. These results support the hypothesis that chaperonin stabilizes the cell membrane by localizing there; however, that is at any temperature not only upon heat shock. Further investigation is required to elucidate heat shock specific mechanism stabilizing the cell. One of the potential mechanism is related to the change of chaperonin composition upon temperature change (See section C in the last year's report, or section III in this report).

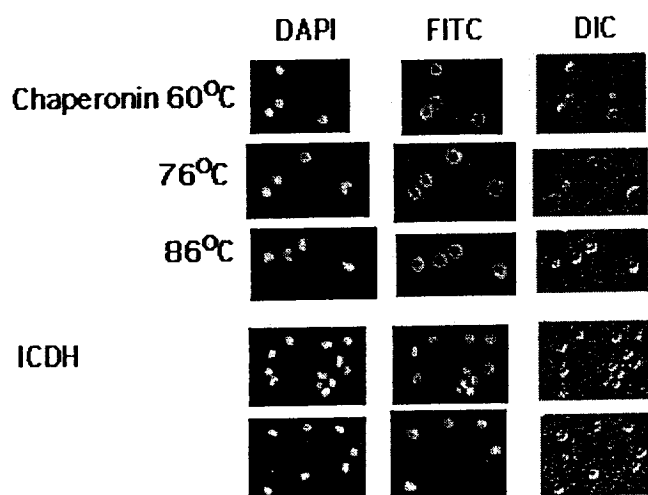


Fig. I-1 Localization of the chaperonin in the cell  
*S. shibatae* culture was grown at 76°C for 3 days. For heat shocked culture, the culture was transferred to 86°C and incubated for 3.5 hrs. For 60°C culture, 1 day-old culture at 76°C was transferred to 60°C and incubated for 7 days to obtain adequate cell density. For the visualization of ICDH, 76°C culture was used.

## II. Liposome Binding

In Section I, the chaperonin from *S. shibatae* was observed to be localized on the cell membrane in vivo. To test affinity of the chaperonin to lipids in vitro, binding of liposomes made from synthetic and natural Archaeal lipids to the purified  $\alpha$ ,  $\beta$ ,  $\gamma$  subunits from *S. shibatae* was investigated. In addition, lipid binding of the chaperonin from *E. coli*, GroEL, and total protein from *S. shibatae* lysate was examined to determine the specificity of the binding.

## MATERIALS AND METHODS

### Liposome Preparation

DPPC and DPPG were purchased from Avanti Polar Lipids. Lipid fraction was purified from *S. shibatae* as follows. Freeze-dried *S. shibatae* cell was extracted with refluxing for 2 x 10 hr with 1:1 of chloroform/methanol solution at 68°C. The extract was filtered with paper and fractionated with C18 column. Elution was done stepwise with 1:1 of methanol/water, 2:5:2 of chloroform/methanol/water, and 65:25:4 of chloroform/methanol/water. The second fraction was used for liposome preparation. Lipid fraction purified from *Halobacterium salinarum* was a gift from Dr. Sprott. For liposome preparation, lipid samples dissolved in organic solvents were dried under the flow of nitrogen gas and resuspended with distilled water or adequate aqueous buffers. The suspension of lipid was sonicated, extruded, and/or loaded onto a gel filtration column to be purified as uniform sized unilamellar liposome.

### Protein Preparation

See Materials and Methods in section III for the preparation of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits. The chaperonin from *E. coli*, GroEL, and co-chaperonin GroES were purchased from Epicentre Technologies. Total protein from *S. shibatae* was prepared as follows. Five milliliters of *S. shibatae* culture grown at 76°C for 7 days was harvested and resuspended with 500  $\mu$ l of 25 mM HEPES (pH7.5) buffer. The cells were opened by sonication and centrifuged at 14,000 rpm for 20 min at 4°C in F45-30-11 rotor (Eppendorf Centrifuge 5810R) to remove the cell debris. Resulted supernatant was centrifuged again with the same condition as liposome should precipitate (at 60,000 rpm for 15 min in TLA 100 rotor (Beckman)) to remove any proteins which could co-precipitate with liposome without binding to it. After the second centrifugation, the supernatant was used as the total protein fraction for liposome binding experiments.

### Liposome Binding

The proteins, liposome, and other components were mixed in the following order to have all the subunits contact each other in equal chance and form the ring complex before contact with liposome, if the condition allowed. Distilled water, a buffer or salt,  $\alpha$  subunit,  $\gamma$  subunit,  $\beta$  subunit, 1 mM ATP/25 mM  $MgCl_2$ , and then liposome was added at the last. The mixture (usually 25  $\mu$ l) was incubated at room temperature for 5 to 60 min unless otherwise described and centrifuged in TLA 100 rotor (Beckman) at 60,000 rpm for 15 min at 20°C. After the centrifugation, the supernatant was removed immediately, and the pellet, which was the liposome fraction, was resuspended with the same volume of buffer as the supernatant. Proteins in the supernatant and pellet were analyzed with SDS-PAGE or Alton gel electrophoresis (see Materials and Methods in section III for Alton gel).

## RESULTS

### Binding to Synthetic Lipids

Liposome made from DPPC, which is neutral in surface charge, did not show significant affinity to the  $\alpha$  subunit (approx. 90% of the protein stayed in the supernatant.) Supposedly forming filaments, the  $\beta$  subunit precipitated in the presence of ATP/Mg before the liposome was added. The protein amount in the pellet did not increase after the addition of liposome; therefore, it was concluded that DPPC liposome did not have affinity either to the  $\alpha$  nor  $\beta$  subunit. When mixture of the  $\alpha/\beta$ ,  $\alpha/\beta/\gamma$ , or the native chaperonin was tested, approx. 60-80% of the protein stayed in the supernatant not binding to the liposome. In contrast, 20:1 mixture of DPPC/DPPG, whose surface charge is slightly negative, showed much better binding to the  $\alpha/\beta$  mixture. Approximately 90% protein precipitated with the liposome in the range of liposome

concentration of 4 to 12 mg/ml (Fig. II-1). This effect was observed either in 20 mM HEPES (pH 7.5) or in 50 mM MES (pH 7.0) buffer.

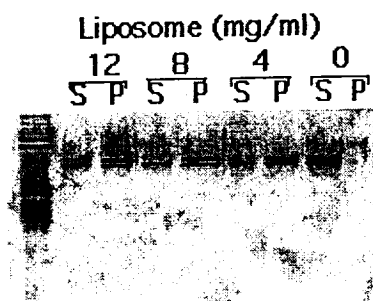


Fig. II-1 Binding of the mixture of  $\alpha/\beta$  subunits to DPPC/DPPG liposome

The supernatant and pellet were analyzed in Alton gel. Lanes S and P represent supernatant and pellet, respectively. Various concentrations (0, 4, 8, and 12 mg/ml) of DPPC/DPPG liposome was tested on binding to the mixture of  $\alpha/\beta$  subunits in 20 mM HEPES buffer.

The binding was then tested under various ionic strengths such as in the presence of  $K_2SO_4$  or NaCl (Fig. II-2). Hundred and 200 mM  $K_2SO_4$  did not inhibit the binding, but 400 and 500 mM  $K_2SO_4$  inhibited it. Approximately 60% and 80% of the protein stayed in the supernatant when 400 and 500 mM  $K_2SO_4$  were present, respectively. The presence of 150 mM NaCl did not interfere with the protein binding to the liposome. When the binding was tested at various pH in MES buffer, pH between 4.72 and 6.85 did not affect it adversely.

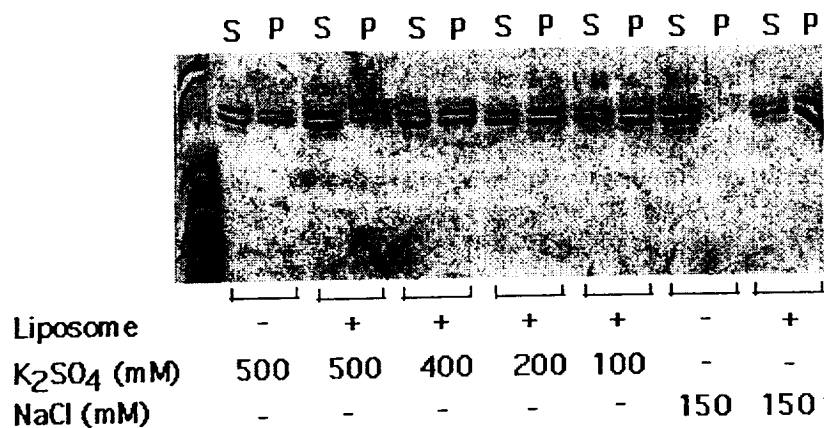


Fig. II-2 Effects of salt concentrations to the binding  
DPPC/DPPG liposome whose concentration was 4 mg/ml was used. The supernatant and pellet were analyzed in Alton gel. Lanes S and P represent supernatant and pellet, respectively.

Preparation of liposome from the lipid extracted from *S. shibatae* is known to provide very low yield (approximately 10%, personal communication with Dr. Sprott). Therefore, the mixture of liposome and aggregation of lipid was used for the binding experiments with the proteins. It showed good binding at 60°C to the  $\alpha/\beta$  (1:1) and  $\alpha/\beta/\gamma$  (1:1:1) mixtures (approx. 90%) but not to the  $\gamma$  subunit alone even when the lipid concentration was estimated as high as 18 mg/ml (Fig. II-3A). In the  $\alpha/\beta/\gamma$  (1:1:1) mixture, only approximately 20% of  $\gamma$  precipitated with the other subunits and the rest stayed in the supernatant, which may suggest only 20% of  $\gamma$  formed the ring complex with other subunits. In case of the lower concentration of lipid (estimated 1.75 mg/ml),  $\alpha/\beta$  (1:1) and  $\alpha/\beta/\gamma$  (1:1:0.1) mixtures showed approximately 50% binding, and  $\alpha/\beta/\gamma$  (1:3:1) mixture did not show significant binding to the *S. shibatae* lipid (Fig. II-3B).

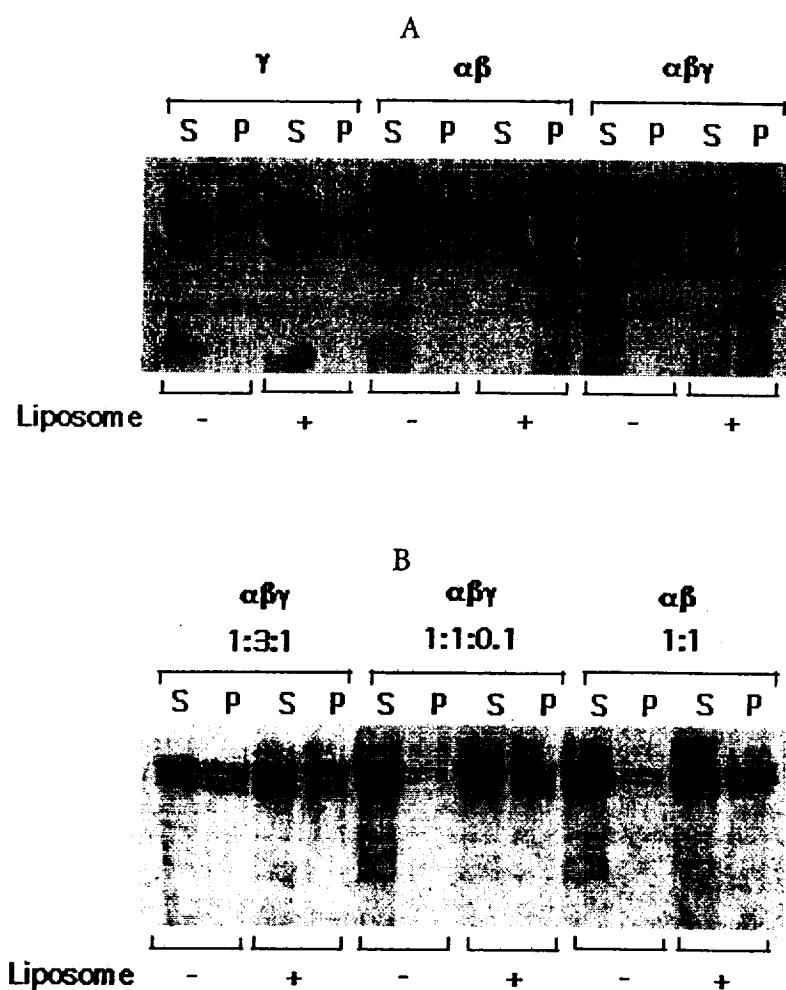


Fig. II-3 Binding of lipid from *S. shibatae*

The supernatant and pellet were analyzed in Alton gel. Lanes S and P represent supernatant and pellet, respectively. A, Lipid from *S. shibatae* was used in the concentration of 18 mg/ml, and protein concentration was 1 mg/ml each of  $\alpha$ ,  $\beta$ , and  $\gamma$ . B, Lipid from *S. shibatae* was used in the concentration of 1.75 mg/ml, and the total protein concentration was 1 mg/ml in each mixture.

In contrast to the lipid from *S. shibatae*, lipid purified from *H. salinarum* is known to provide

liposome in high yield (personal communication with Dr. Sprott). The liposome, in the concentration of 4 mg/ml, bound to the  $\alpha/\beta$  (1:1) and  $\alpha/\beta/\gamma$  (1:1:1) mixtures well (approx. 70-80%) in ATP/Mg dependent manner in the presence of 200 mM  $K_2SO_4$  (Fig. II-4).

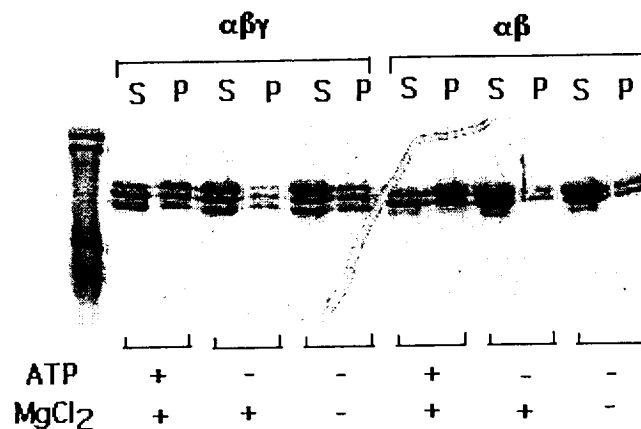


Fig. II-4 ATP/Mg dependent binding of *H. salinarum* liposome. The supernatant and pellet were analyzed in Alton gel. Lanes S and P represent supernatant and pellet, respectively. The total protein concentration was 1 mg/ml in each mixture. Two hundred millimolar  $K_2SO_4$  was present in 50 mM MES buffer (pH7.0).

#### Liposome Binding of Other Proteins

To investigate specificity of the liposome binding of chaperonin subunits, chaperonin from *E. coli*, GroEL, and total protein from *S. shibatae* was tested. Liposome made from DPPC/DPPG (20:1) bound to GroEL effectively (higher than 90%) without requiring ATP or Mg. Addition of the co-chaperonin, GroES, to GroEL did not change the result. Total protein from *S. shibatae* was tested with the liposome made from DPPC/DPPG or *H. salinarum* lipids in the presence of 200 mM  $K_2SO_4$ . Many proteins (more than 15), including the chaperonin, dominantly higher than 30 kD in molecular weight, showed approximately 50% of binding at most to the both kinds of liposomes without requiring ATP or Mg (Fig. II-5).

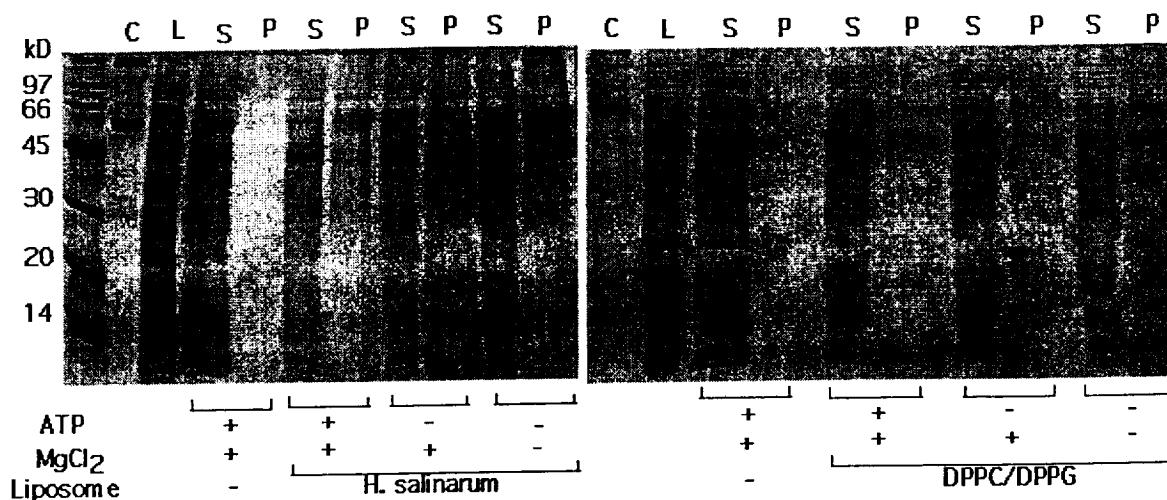


Fig. II-5 Binding of *S. shibatae* lysate to *H. salinarum* and DPPC/DPPG liposomes

The supernatant and pellet were analyzed in SDS-PAGE. Lanes S and P represent supernatant and pellet, respectively. Lanes C represent the chaperonin from *S. shibatae*, and lanes L represent the lysate as a starting material for the binding experiment. Liposome concentration was 4 mg/ml, and the protein concentration was 0.52 mg/ml.

## DISCUSSION

The chaperonin subunits showed binding to charged lipids *in vitro*, which supports the hypothesis that the chaperonin is on the cell membrane and stabilizes it *in vivo*. Since the binding requires ATP/Mg, majority of the bound species may be the ring complex not the subunit monomer. This can explain why GroEL and *S. shibatae* lysate did not require ATP/Mg for the binding; purchased GroEL is already in the form of ring complex, and the chaperonin in the lysate may be present mostly as the ring. It is not known yet how many proteins in the cell other than the chaperonin can bind to the lipid, since the result in Fig. II-5 is not clear if the proteins in the lysate bound to the liposome directly or through the chaperonin. That will be solved by examining the lysate after separating into fractions with or without the chaperonin.

### III. $\gamma$ Subunit of The Chaperonin

We report structural and physiological investigations of the *S. shibatae* chaperonin. The  $\gamma$  gene, its flanking regions, and its deduced amino acid sequence are presented and compared with TF55  $\alpha$  and  $\beta$ . More importantly physiological investigations demonstrate that  $\gamma$  is not a heat shock protein, although it does interact with  $\alpha$  and  $\beta$  to form chaperonin structures. The ratio of  $\alpha$ ,  $\beta$ , and  $\gamma$  in chaperonin is different in cultures grown at 60, 76, and 86°C. The relative stability of the three proteins suggests that that this change in ratio has a functional impact.

## MATERIALS AND METHODS

### *Cloning and Sequencing of Complete $\gamma$ Gene*

Genomic DNA from *S. shibatae* was purified using Qiagen Genomic Tip (Qiagen) and used to amplify the  $\gamma$  gene using the polymerase chain reaction (PCR) method. Previously reported partial PCR fragment sequence of  $\gamma$  gene was used to produce primers (P1: 5'-ATGAACTTAGAGCCTTCCTAT-3' and P2: 5'-TTAACTCCATAAGAACTTGT-3') for the inverse-PCR method (Ochman et al. 1988), which provided the remainder of the  $\gamma$  gene and its flanking regions. Briefly, Ase I-digested genomic DNA was circularized by self-ligation and amplified with Vent polymerase (New England BioLabs). PCR conditions were 30 sec at 94°C, 1 min at 50°C, and 1 min at 72°C, for 25 cycles. Approximately 1.2 kbp fragment was amplified. This fragment was ligated into pBluescript SK(+) (Stratagene) and cloned into *E. coli* (strain DH5 $\alpha$ ) with ampicillin selection, and was sequenced on both strands by the dideoxy-chain termination method (Sanger et al. 1977). Nucleotide and deduced amino acid sequences were analyzed using the program DNASTAR (DNASTAR, Inc.). The complete sequence of  $\gamma$  was submitted to GenBank (accession number AF313410).

### *Expression of the $\gamma$ Gene in *E. coli**

The  $\gamma$  gene was expressed using a plasmid vector pET22b (Novagen) in *E. coli* strain BL21(DE3) codon plus under isopropyl  $\beta$ -D thiogalactopyranoside (IPTG) regulation in LB media



containing 50 mg/ml carbenicillin. (Sigma). The  $\gamma$  gene was amplified from *S. shibatae* genomic DNA by PCR using primers P3 and P4 (P3: 5'-GAAAGAACATATGGCCTATTTATTAAGAGAAGGAACACAG-3' and P4: 5'-TAAAGTACTCGAGAAAACCTAAATAAAATAATCATATCTTAAC-3'), and subcloned into NdeI and XhoI site of the expression vector.

#### *Recombinant Protein Purification*

*E. coli* cells expressing  $\alpha$ ,  $\beta$ , or  $\gamma$  gene from *S. shibatae* were harvested by centrifugation at 7000 rpm for 5 minutes (RC-5, Sorvall, GSA). A freeze/thaw cycle was repeated three times in which the pellet was frozen at  $-80^{\circ}\text{C}$ , and then thawed at  $21^{\circ}\text{C}$ . One milliliter of protease inhibitor cocktail for bacterial cells (Sigma) was added per 4 g of cells in the first cycle while the pellet was frozen. After the freeze/thaw cycles, twice volume of buffer A (25 mM HEPES, pH 7.5), lysozyme (46,400 u/ml in final, Sigma), and benzonase (1 u/ml in final, Sigma) were added to the cell pellet, which was then allowed to sit on ice for 30 minutes. The cell suspension was sonicated for 60-70 min with the power set to 3 and the duty cycle set to 40% (Branson Sonifier 450) with 2 min intervals every 10 min by removing the sonicator tip from the suspension to cool down. The lysate was centrifuged at 18,000 rpm for 10 minutes, and the second sonication of the resulting pellet was repeated if necessary. The supernatant was then heated to precipitate heat labile proteins from *E. coli*. Lysate of  $\alpha$  and  $\beta$  expressing cells were heated to  $86^{\circ}\text{C}$  for 30 minutes. Lysate of  $\gamma$  expressing cells were heated to  $73^{\circ}\text{C}$  for 30 minutes. The lysate was cooled on ice and was then centrifuged at 30,000 rpm for 30 minutes (45Ti rotor, Beckman). The supernatant was fractionated by anion exchange chromatography (DEAE-Sepharose fast flow, 140 ml bed volume, Pharmacia) and was eluted using a 10 column volume of linear NaCl gradient from 0 to 800 mM in buffer A. Fractions containing the expressed protein (detected by SDS-PAGE, 8-16% gradient) were pooled, concentrated with a centrifugal concentrator (Centricon Plus-80, 10K MWCO), diluted with buffer A to 50 ml, and refractionated with anion exchange chromatography (Mono-Q 10/10, Pharmacia) and was eluted using a 26 column volumes of linear NaCl gradient from 0-400 mM in buffer A. Fractions containing the expressed protein (detected by SDS-PAGE, 8-16% gradient) were pooled, filter sterilized (0.2  $\mu\text{m}$  syringe filter), concentrated to a volume of 200  $\mu\text{l}$  with centrifugal concentrators (Centricon 30, 30K MWCO) and washed three times with 2 ml of filter sterilized buffer A. Protein concentration was determined by using DC protein assay kit (Biorad) with BSA as standard. The final fraction was stored in small aliquots at  $-80^{\circ}\text{C}$ .

#### *Native Chaperonin Purification*

For obtaining  $60^{\circ}\text{C}$  culture of *S. shibatae*, the cell was cultivated at  $76^{\circ}\text{C}$  overnight then transferred to  $60^{\circ}\text{C}$  and cultivated for approximately 10 days until the cell density reached about  $2 \times 10^8$  cells/ml. For  $76^{\circ}\text{C}$  culture, the cell was cultivated at  $76^{\circ}\text{C}$  for approximately 4 days. For  $86^{\circ}\text{C}$  culture, the cell was cultivated at  $76^{\circ}\text{C}$  to log phase then transferred to  $86^{\circ}\text{C}$  for 20 hr. Native chaperonin from those cultures was purified by following the same procedure as the recombinant protein with the following modifications. Ten millimolar each of  $\text{MgCl}_2$  and KCl were added to the buffer A to stabilize the chaperonin complex, and the heating step to remove proteins from *E. coli* was omitted. The fraction pooled after DEAE column was concentrated and loaded onto 30 ml of 10-30% linear glycerol gradient and centrifuged at 16,000 rpm for 17 hr at  $4^{\circ}\text{C}$  in SW28 rotor (Beckman) before loaded onto Mono-Q column, if necessary.

#### *Northern Hybridization*

*S. shibatae* cells were grown at  $76^{\circ}\text{C}$  to log-phase (approx.  $3 \times 10^8$  cells/ml), and 1-5 ml of the culture was used per lane for electrophoresis. Total RNA from the culture was purified by using

RNeasy kit (Qiagen). Amount of RNA to be loaded on the gel was normalized by absorbance at 260 nm after DNase treatment was performed. The RNA samples were separated in 6.6% formaldehyde denaturing 1% agarose gel buffered by HES (50 mM HEPES, 1 mM EDTA, and 5 mM sodium acetate, pH 7.0). After the gel electrophoresis, RNA was transferred onto a nylon membrane by capillary blotting with 20 x SSC. PCR products from the  $\alpha$ ,  $\beta$ , and  $\gamma$  gene (regions 167-1003, 187-1527, and 359-1183 bp from the starting codon in each gene, respectively) were used as probes for the hybridization after labeled with horseradish peroxidase by using ECL kit (Amersham). Hybridization and signal development were carried out by following the instruction for the kit. The signal was detected on X-ray film, and the film was used for quantification of the signal.

#### *Native and Alton Gel Electrophoresis*

Four to 12% gradient polyacrylamide (acrylamide:bis 37.5:1, Amresco) gel without SDS was used for native gel electrophoresis. Alton gel was prepared by adding 5.4 g of urea to 15 ml of separation gel solution of regular SDS denaturing 10% polyacrylamide gel system buffered with Tris. Quantification of the bands was performed by using an image acquisition system (BioChem, UVP) and analysis software (LabWorks 4.0, UVP).

#### *Western Blotting*

Proteins separated in Alton gel electrophoresis were transferred to a nitrocellulose membrane electronically (Trans-blot, BioRad), and the transfer was confirmed by Ponceau S staining of the membrane. The membrane was blocked with 5% non-fat dry milk in PBS-T for one hour, washed with PBS-T for 5 min twice, and crossreacted for one hour with 100,000 times diluted rabbit serum which contains polyclonal antibody raised against the chaperonin complex purified from *S. shibatae*. After 4 times wash with PBS-T for 10 min each, the membrane was crossreacted with 50,000 times diluted peroxidase-labeled goat anti-rabbit IgG (affinity purified, 1.0 mg/ml, Kirkegaard & Perry Laboratories Inc.). Following 4 times of 10 min wash with PBS-T, signal was developed by using ECL kit (Amersham) and detected on X-ray film. All the wash and crossreactions were carried out at room temperature.

#### *ATPase Assay*

Ten micrograms of a mixture of the  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits, 10  $\mu$ g of the chaperonin, or 10  $\mu$ g of GroEL (Epicentre Technologies) was added to 40  $\mu$ l of reaction buffer (50 mM HEPES (pH7.5), 30 mM KCl, 1 mM DTT, 0.5 mM magnesium acetate, 0, 200 mM, or 1 M ammonium sulfate, and 4 mM ATP in final concentration). The reaction was carried out for 15 min at 70°C for the *S. shibatae* proteins and at 37°C for GroEL. The phosphate product upon the reaction was detected by using malachite green by following Lanzetta's method (Lanzetta et. al., 1979) with the following modification. Triton X-100 (0.1% in the MG/AM/Sr solution) was used instead of Sterox. The developed color was compared with that of phosphate standards, which were 0-0.25 mM of KH<sub>2</sub>PO<sub>4</sub> solutions, to quantify the amount of reaction product. Assay without proteins was performed at each reaction temperature and subtracted as background.

#### *Differential Scanning Calorimetry (DSC)*

All DSC studies were carried out using a VP-DSC ultrasensitive differential scanning calorimeter (MicroCal, LLC, Northampton, Ma) Denaturation studies of the  $\alpha$ ,  $\beta$  and  $\gamma$  subunits both alone and in mixtures assembled into rings were carried out in 25 mM HEPES, pH 7.5. To determine the penetration temperatures,  $T_m$ , of the individual subunits, separate 1.5 mg/ml (approx. 0.017 mol/l) solutions of the proteins were individually scanned from 25-110°C at a rate of 50 deg/hr (Fig. III-7A). For studies of the effects of  $\gamma$  incorporation into rings, rings were assembled with

and without  $\gamma$  using the appropriate weight ratios determined by densitometry analysis of quantitative PAGE. Rings without  $\gamma$  (1:1 ratio of  $\alpha$  to  $\beta$ ) were prepared by the addition of ATP/Mg (1 mM ATP, 25 mM MgCl<sub>2</sub>) to a 1 ml sample containing 1.5 mg/ml  $\alpha$  and  $\beta$ . The sample was incubated at 60 degrees C for 1 hr to promote ring assembly, and scanned from 25-110 degrees C, at a rate of 50 deg/hr versus a reference likewise containing ATP and Mg. In a similar method, rings containing  $\gamma$  were assembled using the ratio 1:1:0.1  $\alpha$   $\beta$   $\gamma$  respectively by adding ATP/Mg to a 1 ml solution containing 1.5 mg  $\alpha$  and  $\beta$ , and 0.15 mg  $\gamma$ . The sample was incubated as before to promote assembly, and scanned versus buffer containing ATP/Mg. Data for the penetration of rings are represented in Fig. III-7B.

DSC data processing was performed using the program Origin (MicroCal, LLC, Northampton, Ma). In each case, buffer-buffer reference scans were subtracted from the sample scans, and appropriate baselines were fitted.  $T_m$  and  $\Delta H$  values were calculated by fitting the simplest two-state curve using the Levenberg-Marquardt non-linear least squares method.

## RESULTS

### *The $\gamma$ gene*

To explore  $\gamma$  expression we first cloned and sequenced the complete *S. shibatae*  $\gamma$  gene and its flanking regions (Fig. III-1). This was accomplished using the partial sequence information reported by Archibald and coworkers (Archibald et. al., 1999) to design DNA primers used for standard and inverse PCR techniques. In the DNA fragment obtained by PCR we found a 1608 bp open reading frame with 38.8% G+C, comparable to the overall 34.6% G+C of all known *S. shibatae* genes (Grogan et. al., 1990). We identified this open reading frame as the  $\gamma$  gene by its homology to published sequences (Archibald et. al., 1999). In the flanking region upstream from the assumed start codon for the  $\gamma$  gene we found a hexanucleotide sequence (5' TTTATA3') that precisely fits the consensus for the archaeal box A promoter element ((C/T)TTA(T/A)A) (Zillig et. al., 1993). In addition, we found an eight-bp region in the vicinity of the box A, which fits the consensus for the so-called BRE promoter element with only one mismatch (Bell et. al., 1999). A candidate transcription start (purine following a pyrimidine) is located in the prescribed 25-28 bp from the box A (Zillig et. al., 1993), but this was not confirmed. We found no obvious Shine-Dalgarno sequence (5' TGAGGTG3') for the  $\gamma$  gene, but it is reported that many archaeal genes lack this sequence (Zillig et. al., 1993).

ATTATGTTCTTCCGCTATATATTTGCTCTGACAAATGACACAAAACAGTGGCTGCTGCTAGCTGACATTTTAACTCTACACT 90  
 ATTTTATTTCTACTGTTAGATCTAGTAAACACAAATTTTATTTCTTTTCTAAACCTAGTACAGTGGAGTGAACAAAACATGGCTT 180  
 M A  
 ATTTATTAAAGCAAGCAACACGACCTCTACTGCAAAACAGGTAACTAAACACACTAGCTGTAGCAAAATATTACIGCAATGCTAA 270  
 Y L L R E G T Q R S T G N E V I L N N I A V A K I L L E M L  
 AGTCAAGCTTAGGCTCTAAAGCTTTAGCAACCTGCTAGCTGACGGGCAACACCTTACATTAACCTATGACGGTGGACATAGTTAA 360  
 K S S L G P K G L D K M L V E G Q D I T I T N D G A T I V K  
 ACATGCAAGTCCAGCATCTCTACTGCAAAATTAATCTATTGAAACCTGTAACCTGCTGCTAGCTGACGGTGGACATAGTTAA 450  
 N M E V Q H P T A K L L I E T A K T V D T E V G D G T T S V  
 TGGTCTCTGCTGCTTACTATTAGAAAAGCTGAGCTTTTCTGATTCGCAAGTCTCTGCTAGCTGCTGCTATAGTACGGTATAGG 540  
 V V L A G L L L E K A E D L L N Q K I H P T V I I E G Y R K  
 CTTAAGTCTCTCTTACTATTGTAAGAAAGCTTTCGACATAGCTTATGCTGACCTAGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 630  
 A L S S S L E L L K S I A D K I S P E D R K I V H D L V Y T  
 CTTCTGCT 720  
 T L S S K F F S T E H T L E K I I N L V I E A S L A V L D K  
 GACATGCAAGCTTAGGCTCTAAAGCTTTAGCAACCTGCTAGCTGACGGGCAACACCTTACATTAACCTATGACGGTGGACATAG 810  
 R D G T Y D L D I K N I K I V K V N G G E F D D S E L V N G  
 TGGTCTCTGCTGCTTACTATTAGAAAAGCTGAGCTTTTCTGATTCGCAAGTCTCTGCTAGCTGCTGCTGCTGCTGCTGCTGCTGCT 900  
 I V V D K E P T N E N M P K R A E N V K V M L A D F P L K L  
 AAAAAACCAATTAAGCTGACCTGCT 990  
 E K T E I S M K L G I S D P T Q I K G Y L D E Q T A Y V K Q  
 TGGTCTCTGCTGCTTACTATTAGAAAAGCTGAGCTTTTCTGATTCGCAAGTCTCTGCTAGCTGCTGCTGCTGCTGCTGCTGCTGCT 1080  
 M V D K I K A M G V K L F I T Q K D I D E V A S Y L M G K S  
 GCTATTAAGCTTAGGCTCTAAAGCTTTAGCAACCTGCTAGCTGACGGGCAACACCTTACATTAACCTATGACGGTGGACATAG 1170  
 G I I A L K N V K R S D I E L L S R A T G A K I A S S M K D  
 CTATTAAGCTTAGGCTCTAAAGCTTTAGCAACCTGCTAGCTGACGGGCAACACCTTACATTAACCTATGACGGTGGACATAG 1260  
 A N E S D L G E A K L V E V R N L G K N K Y L F I Q S D K A  
 AAGCT 1350  
 K A V T V I I K G S N N M V T D E A E R S L N D A F N S I R  
 ACTGCT 1440  
 N L L L E P Y I V A G G G A V E E E L A K R L R E N A G K V  
 CCGCAAGCTTAGGCTCTAAAGCTTTAGCAACCTGCTAGCTGACGGGCAACACCTTACATTAACCTATGACGGTGGACATAG 1530  
 P G K E Q L A F N A F A D A L E E Y V S I L S E T A G M D P  
 TAAGTGGCTTAGGCTCTAAAGCTTTAGCAACCTGCTAGCTGACGGGCAACACCTTACATTAACCTATGACGGTGGACATAG 1620  
 I S A L T E I R H K H A N G L K N A G I D I V K A R I Y D N  
 TGGTCTCTGCTGCTTACTATTAGAAAAGCTGAGCTTTTCTGATTCGCAAGTCTCTGCTAGCTGCTGCTGCTGCTGCTGCTGCTGCT 1710  
 M L E L K V I D S L K V K E Q V L K S A T E A A T A I L K I  
 ACGACTGCT 1800  
 D D M I A A A P A K Q Q P Q P Q Q P N P Y L G  
 TTTATTTAGCTTTCTTTATTTACTTTATGCACTTAAATATCTGTTTCAAGCTTTTCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 1890  
 TGAGATCTACTCCAGGCT 1980  
 TAATGCT

Fig. III-1. DNA and deduced amino acid sequences of the  $\gamma$ -subunit. The 1608 bp coding for 535 amino acid protein is flanked upstream by an archaeal promoter element known as box A (box at position 43 bp) and downstream by an oligo T region typical of archaeal transcription terminators (dotted at position 1638 bp).

The  $\gamma$  gene codes for a 535 amino acid protein with a predicted molecular mass of 58.54 kDa and an isoelectric point of 5.5. The  $\gamma$  protein is rich in hydrophobic residues, but lacks tryptophan

and cysteine. Like other  $\gamma$  proteins, it is closely related to TF55  $\alpha$  and  $\beta$ . The *S. shibatae*  $\gamma$  protein is overall 54% identical to  $\alpha$  and 43% identical to  $\beta$  (Fig. III-2). (The sequence identity between  $\alpha$  and  $\beta$  is 54%.)  $\gamma$  is smaller than both  $\alpha$  and  $\beta$  with 25 fewer amino acids than  $\alpha$  and 17 less than  $\beta$ . Comparing all three proteins,  $\gamma$  and  $\alpha$  are missing an eleven amino acid sequence that is present at the N-terminus of  $\beta$ , while  $\gamma$  and  $\beta$  are missing a six amino acid insert in the intermediate domain and a nine amino acid extension at the C-terminus of  $\alpha$  (Fig. III-2).

1	MAS.....PVLLKEGTSRTTGRDALRNNILAAKTLAEMLRSSLG	Alpha
1	MATATVATTPEGI PVII LKEGSSRTYGEALRANI AAVKAI EEALKSTYGS	Beta
1	MA.....YLLREGTORSTGNEVI LNNI AVAKI LLEMLKSSLG	Gamma
41	PKGLOKMLI DSFGDVTI TNDGATIVKDMEI DHPAAKLLVBAAKADDAEVG	Alpha
51	PRGMDKMFVDSLGDI TI TNDGATILDKMDLQHPTGKLLVQI AKGQDEETA	Beta
38	PKGLOKMLVEG- QDI TI TNDGATIVKNMEVQHPTAKLLI ETAKTVDT EVG	Gamma
91	DGTTSAVVL AGALL EKAESLLDQNI HPTITIEGYKKAYTKALELLPOLGT	Alpha
101	DGKTAVI LAGELAKKAEDLLYKEI HPTIIVSGYKKAEELAKTIQDI AQ	Beta
87	DGTTSMVVL AGLLLEKAEDLLNQKI HPTVII EGYRKALSSSLELLKSI AD	Gamma
141	RIDIRDLNS SVARDT LRKI AFITTLASKFI AEGAEINKIIDMMI DAI VNVA	Alpha
151	PVSI ND.....TDVLRKVALTSLGSKAVAGAREY- LADLVKAVAQVA	Beta
137	KISPED.....RKI VHDLVYTTLSSKEFST EHTLEKII NLVI EASLAVL	Gamma
191	EPLPNEGYNVSLDLI KIDKKKGGSI EDSVLVKGLVLDKEVWHPGMPRRVT	Alpha
193	E LRGDKWYVDLDVQI VKKHGGSI NDTQLVYGI VVDKEVWHPGMPKRI E	Beta
181	D- KROBT VDLDI KNI KIVKNGGEF DSELVNGI VVDKEPTNENMPKRAE	Gamma
241	KAKIAMLDALEVEKPEISAKI SITSPEDIKAF LDEESKYLKDMDVKLAS	Alpha
242	NAKIALLDASLEVEKPELDAEL RINDPTOMHKF LEEBENLKEKVDKI AA	Beta
230	NKVM LADF PLKLEKTEISMKLGIS DPTQIKGYLDEDTAYVKQMDVKI KA	Gamma
291	IGANVVI CQKGI DDI AQHF LAKKGI LAVRRVKRSDIEKLEKALGARI ISS	Alpha
292	TGANVVI CQKGI DEVAQHY LAKKGI LAVRRAKKSDLEKLARATGGRVLSN	Beta
280	MGVKLFITQKDI DEVASYLMGKSGI I ALKNVKRSDIELSRATGAKI ASS	Gamma
341	IKDATPDOLGYAE LVEERRVGNCKMVFIEGAKNLKAVNI LLRGSNDMAL D	Alpha
342	IDELTSQDLGYAALVEERKVGEDKMVFIEGAKNPKSMVSI LIRGGLERVVD	Beta
330	MKDANES DLGEAKLVEVRNLGKNKYLFIQSDKA- KAVTVI LKGSNNMVT D	Gamma
391	EAERSI NDALHAL RNI LLEPVI LPGGGAI EL ELAMKLREYARSVGGKEQL	Alpha
392	ETERALRDALGTVADVI RQGRAVAGGGAVEI EL AKRLRKYAPOVGGKEQL	Beta
379	EAERSLNDAFNSI RNLLLEPYI VAGGGAVEE EL AKRLRENAGKMPGKEQL	Gamma
441	AI EAFADALEEI PTI LAETAGLEAI SALMDL RARHAKGLTN- TGVQVI GS	Alpha
442	AI EAYANAI EGLIM LAENAGLOPI DKLMQL RSLHEKETNKWYGLNLFT G	Beta
429	AFNAFADALEEYVSI LSETAGMOPI SALT EI RHKHANGKNI AGI DI VKA	Gamma
490	KIVDDVYALNI I EPI RVKADVLKSATEAATAILKI DDLI AAAPL KSEKK G	Alpha
492	N- PEDMMKLGVI EPALVKMNAI KAATEAVTLVLRI DDI VAAGKKGGSEPS	Beta
478	RITYDNMLEL KVIDSLKVKE DVLKSATEAATAILKI DDM AAAPAKQQPQ-	Gamma
540	GGSKKEESGGEGGAGTPSLGD	Alpha
541	GKKEKEEKSSSE- - - - - D	Beta
527	- - - PQQPNPYL- - - - - G	Gamma

Fig. III-2. Amino acid sequence comparison among  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits. Identical amino acids between at least two of the proteins are boxed.

These sequence comparisons clearly indicate a relationship between  $\alpha$ ,  $\beta$ , and  $\gamma$  but our goal was to determine their physiological and structural relationships. The concentrations of TF55  $\alpha$  and  $\beta$  are known to increase in cells exposed to heat shock and to be associated with the double-ring structure known as a chaperonin (Kagawa et al. 1995). We therefore investigated if  $\gamma$  is physiologically related to  $\alpha$  and  $\beta$  (i.e., its synthesis is increased in response to high temperature stress) and whether it interacts with  $\alpha$  and  $\beta$  in forming the chaperonin structure.

#### *Temperature-dependent $\gamma$ expression*

To explore the physiological relationship between  $\alpha$ ,  $\beta$ , and  $\gamma$  or more specifically to determine if  $\gamma$  is a heat shock protein, we studied the relative expression of  $\alpha$ ,  $\beta$ , and  $\gamma$  at different temperatures. Using DNA probes designed to distinguish between the three genes, we compared their relative mRNA levels in cells exposed to temperatures ranging from 60-90°C and measured how quickly  $\gamma$  mRNA levels change (Fig. III-3).

Shifting mid-log phase cultures grown at 79°C to the heat shock temperature of 86°C for 30 min., showed a significant increase in both  $\alpha$  and  $\beta$  mRNA levels as expected (Kagawa et al. 1995) (Fig. III-3A). Under these conditions  $\gamma$  mRNA, however, appeared to decrease to negligible levels (Fig. III-3A). In contrast, shifting the 79°C-grown cultures to 60°C for 30 min. caused a 60% drop in  $\alpha$  and  $\beta$  mRNA levels, but a noticeable increase in  $\gamma$  mRNA levels. These results suggested that  $\gamma$  expression is cold, rather than heat, induced.

To further characterize the temperatures-dependent expression of  $\gamma$ , we cultivated *S. shibatae* at 60°C for 20 hrs, shifted cells to temperatures ranging from 60 to 90°C, and measured mRNA levels after 30 min. (Fig. III-3B). These experiments confirmed that  $\gamma$  mRNA levels are highest at lower temperatures. In comparison  $\beta$  mRNA levels are relatively low between 60 and 72°C and peaked between 83 and 86°C (heat shock). Since northern analysis measures only the abundance of specific mRNAs and since  $\gamma$  mRNA levels were highest at the minimum cultivation temperature (60°C) the changes observed at higher temperatures represent some combination of increased degradation and decreased synthesis. At the heat shock temperature (86°C), however,  $\gamma$  mRNA levels significantly decreased within 30-min. This indicates that at this heat shock temperatures the cells actively deplete the  $\gamma$  mRNA.

To determine how rapidly *S. shibatae* increases  $\gamma$  mRNA levels in response to a shift to low temperature, we transferred cells grown at 76°C to 60°C and monitored  $\gamma$  mRNA for 60 min. (Fig. III-3C). We observed that relative to 76°C, at 60°C mRNA levels had nearly doubled within five min. and had increased approx. 10-fold by 60 min. This indicates that cells quickly adjust  $\gamma$  mRNA at low temperatures. In contrast during heat shock at 86°C  $\alpha$  and  $\beta$  mRNA levels increase between 5 and 10 fold in 30 min., but decline again to 75°C-control levels within 60 min. (data not shown).

These results establish that  $\gamma$ , like  $\alpha$  and  $\beta$ , is temperature regulated, although unlike  $\alpha$  and  $\beta$ ,  $\alpha$  and  $\beta$ , is not a heat shock protein by the criterion that high temperatures stimulate its synthesis. To determine if  $\gamma$  interacts with  $\alpha$  and  $\beta$ , we investigated the double-ring structures these proteins are known to form (Kagawa et al. 1995).

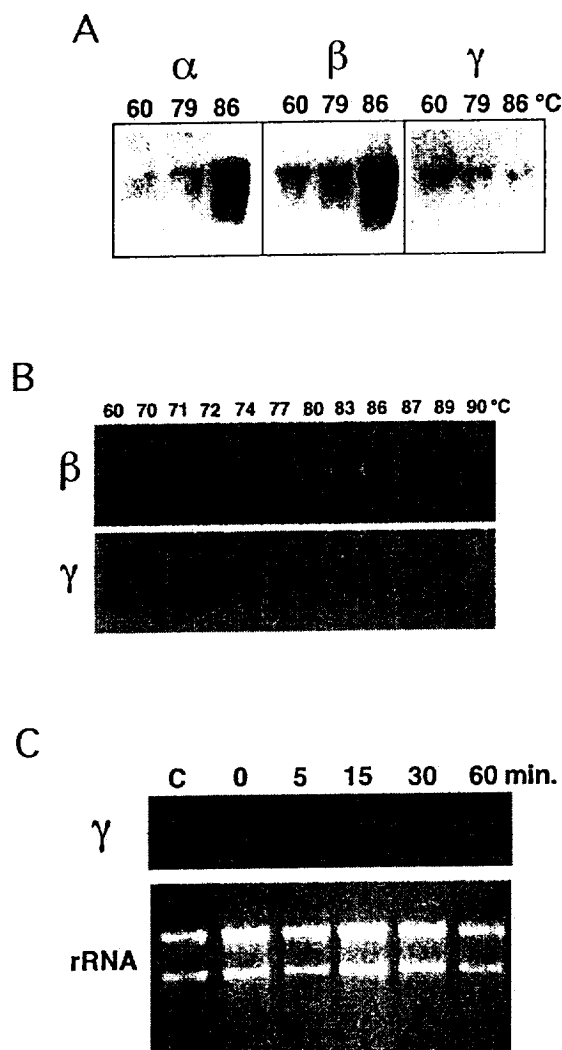


Fig. III-3 Low temperature specific expression of the  $\gamma$  gene.

A, *S. shibatae* culture grown at 79°C was transferred to 60 and 86°C for 30 min, and total RNA was purified and analyzed by Northern hybridization with specific probes to the genes, respectively. B, *S. shibatae* culture grown at 76°C then incubated at 60°C for 20 hr was transferred to the temperatures shown in the figure for 30 min, and total RNA was purified and analyzed by Northern hybridization. C, *S. shibatae* culture grown at 76°C was cooled down to 60°C and incubated at the temperature for the indicated time length. Lane C indicates the culture before cooled down as a control. Total RNA was purified and analyzed by Northern hybridization. Lower panel shows that the same amount of total RNA was loaded on each lane judged by the intensity of rRNA bands stained by ethidium bromide.

#### *The composition of the double rings*

It is reported that mixtures of purified  $\alpha$  and  $\beta$  in the presence of ATP/Mg associate to form 18-membered double-ring structures, referred to as chaperonins (Kagawa et al. 1995) and that chaperonins have a characteristic mobility in native PAGE (Yaoi et al., 1998). To determine if  $\gamma$  is

a constitutive subunit of chaperonins, we analyzed  $\alpha$ ,  $\beta$ , and  $\gamma$  mixtures by native and denaturing PAGE (Fig. III-4). Using native PAGE we observed that the purified recombinant  $\alpha$ ,  $\beta$ , and  $\gamma$  proteins have distinctive electrophoretic mobilities (Fig. III-4A, lanes 1-3). We also observed that a 1:1:1 mixture of  $\alpha$ ,  $\beta$ , and  $\gamma$  in the presence of ATP/Mg did assemble into a higher-order structure, indicated by the appearance of a chaperonin band (Fig. III-4A, lane 4). The persistence of  $\gamma$  and  $\alpha$  bands diminished and the  $\beta$  band disappeared. The chaperonin had a different electrophoretic mobility from pure  $\alpha$  or pure  $\beta$  chaperonins (Fig. III-4A, compare lanes 4, 5, and 6). Notably, pure  $\gamma$  in the presence of ATP/Mg did not form a chaperonin structures (Fig. III-4A, lane 7).

Native PAGE suggested that there are  $\alpha$ ,  $\beta$ , and  $\gamma$  containing chaperonins by a slight shift in electrophoretic mobility. The presence of all three proteins in the chaperonin was confirmed by excising the chaperonin band from the native polyacrylamide gel and analyzed the associated proteins using a special denaturing PAGE system we devised (Fig. III-4B).

To separate  $\alpha$ ,  $\beta$ , and  $\gamma$ , which have nearly identical mobilities in standard (SDS) denaturing-PAGE, we developed a urea-SDS-PAGE system referred to as "Alton-PAGE" (see Materials and Methods for details). This system clearly separates the three proteins, indicated by purified proteins as standards (not shown) and confirmed that indeed they are all present in the chaperonin complex that formed in a 1:1:1 mixture of the proteins (Fig. III-4B).

Under the in vitro conditions we used, however, the three proteins do not associate with 1:1:1 stoichiometry;  $\gamma$  is a relatively minor component compared to  $\alpha$  and  $\beta$ . This was indicated by the results of Alton PAGE and by the presence of  $\alpha$  and  $\gamma$  subunits in native polyacrylamide gels (cf. Fig. III-4A, lane 4 and Fig. III-4B). The disproportionate incorporation of the three proteins in chaperonins suggested that their ratios may impact their stabilities.



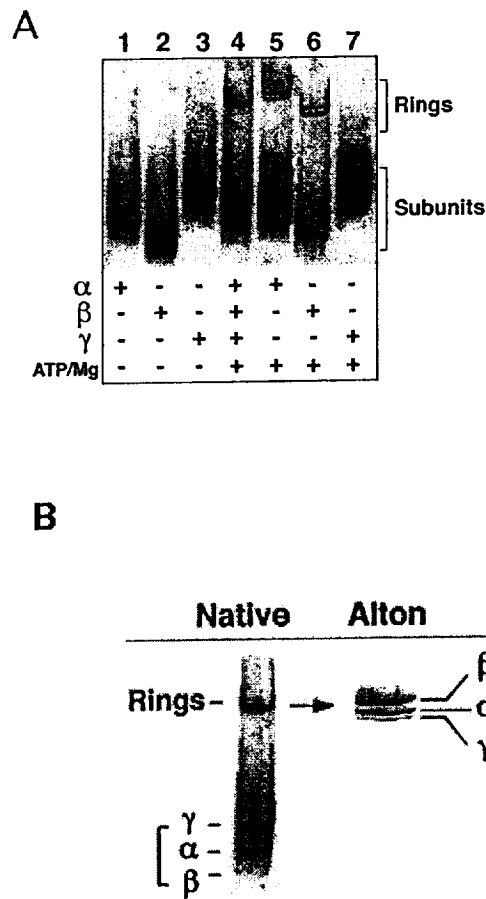


Fig. III-4 Incorporation of the  $\gamma$  protein in ring complex.

A, Recombinant  $\alpha$  or  $\beta$  protein was incubated at 76°C, and the  $\gamma$  protein was incubated at 60°C in the presence of 1 mM ATP and 25 mM  $\text{MgCl}_2$  for 1 hr (lanes 5, 6, and 7). Concentrations of ATP and  $\text{MgCl}_2$  are same in all the incubations unless otherwise described. Equimolar mixture of the  $\alpha$ ,  $\beta$ , and  $\gamma$  proteins was incubated at 60°C in the presence of ATP/Mg for 1 hr (lane 4). The same mixture incubated at 75°C showed an identical pattern (data not shown). Lanes 1, 2, and 3 show the  $\alpha$ ,  $\beta$ , and  $\gamma$  proteins before incubations. B, Equimolar mixture of recombinant  $\alpha$ ,  $\beta$ , and  $\gamma$  proteins was incubated at 75°C in the presence of ATP/Mg for 1 hr, and the band representing ring complex was separated in the native gel and excised. The excised gel piece was heated in the minimum volume of Laemmli buffer for denaturation of the proteins and loaded on Alton gel to visualize the  $\alpha$ ,  $\beta$ , and  $\gamma$  proteins.

#### ATPase activity

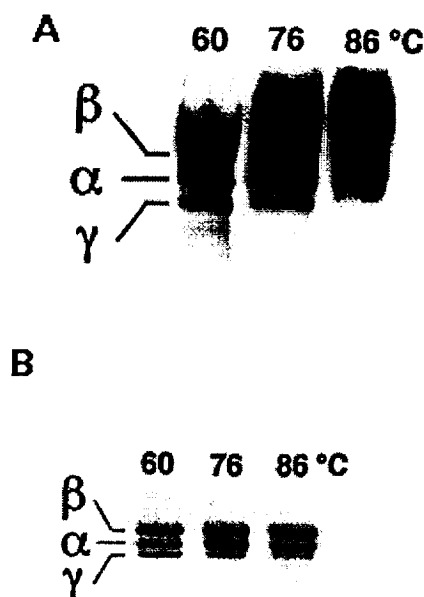
The chaperonin and the mixture of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits did not show detectable ATPase activity in the absence of ammonium sulfate. However, they showed detectable but weak ATPase activity (approx. 4% of that of GroEL) only in the presence of 1M ammonium sulfate (data not shown). This observation is consistent to a result from the chaperonin from *Methanopyrus kandleri* reported by Andra and coworkers (Andra et. al., 1998)

#### Temperature-dependent ratios of $\alpha$ , $\beta$ , and $\gamma$

In vitro reconstitution experiments suggested possible interactions between  $\alpha$ ,  $\beta$ , and  $\gamma$  but our goal was to reveal the relationship between these proteins in vivo. Our northern analyses had revealed the temperature-dependence of  $\alpha$ ,  $\beta$ , and  $\gamma$  gene expression, so we studied the proteins in cultures grown at different temperatures (Fig. III-5).

Cultures grown at 60, 76, and 86°C to late-log phase (approx.  $2.0 \times 10^9$  cells/ml) were extracted for total proteins and analyzed directly by Alton-PAGE or used to purify chaperonins by chromatography. The presence of  $\alpha$ ,  $\beta$ , and  $\gamma$  were identified by immunoblotting Alton-polyacrylamide gels using a polyclonal antibody raised against the *S. shibatae* chaperonin (Fig. III-5A). The antibody detectable  $\alpha$ ,  $\beta$ , and  $\gamma$  in cells cultivated at 60 and 76°C, but only  $\alpha$  and  $\beta$  in cells cultivated at 86°C, i.e., under heat shock condition. We determined that the antibody did not quantitatively react with the three proteins, however, so it did not indicate their relative abundances at these different temperatures.

We purified chaperonins from cultures grown at 60, 76, and 86°C and determined the relative abundance of  $\alpha$ ,  $\beta$ , and  $\gamma$  as their constitutive subunits using Alton-PAGE (Fig. III-5B). As expected from the northern analysis, the proportion of  $\gamma$  was highest at 60°C, approx. equaling  $\alpha$ , but lower than  $\beta$ . At 76°C the proportions of  $\alpha$  and  $\beta$  were approximately equal and  $\gamma$  was a minor constituent. At 86°C  $\alpha$  and  $\beta$  proportions were again similar but  $\gamma$  was not detectable. The actual percentages of  $\alpha$ :  $\beta$ :  $\gamma$  determined by an image acquisition system (UVP) were 60°C = 20:61:19; 76°C = 41:56:3; and 86°C = 41:59:0. These results reveal that at all temperatures  $\beta$  remains approximately the same and that  $\alpha$  and  $\gamma$  percentages change most dramatically at low temperatures.



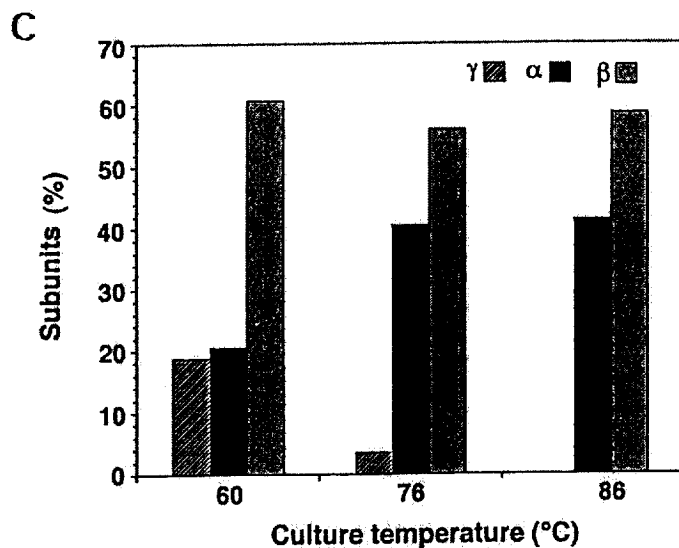


Fig. III-5 Ratio of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits in vivo at different temperatures.

A, Total proteins from *S. shibatae* cultures grown at 60, 76, and 86°C were separated in Alton gel and analyzed by Western blotting. Five micrograms each of total protein from the 60 and 76°C samples, and 2.5  $\mu$ g from 86°C sample were loaded.

B, The chaperonin was purified from *S. shibatae* cultures grown at 60, 76, and 86°C and analyzed in Alton gel. Three micrograms each of the protein was loaded.

C, The bands representing alpha, beta, and gamma subunits in the gel shown in III-5B was quantified.

#### *Thermostability of $\alpha$ , $\beta$ , and $\gamma$*

$\gamma$  is incorporated into chaperonins at low temperatures and absent at heat shock temperatures.

To investigate the structural consequence of this we determine the denaturation temperatures of  $\alpha$ ,  $\beta$ ,  $\gamma$ , and chaperonins containing all three proteins or only  $\alpha$  and  $\beta$  using differential scanning calorimetry (Microcal) (Fig. III-6). We observed that  $\gamma$  denatured at 80.5°C,  $\alpha$  at 95.7°C, and  $\beta$  at 96.7°C (Fig. III-6A). The difference in the stability of chaperonins containing all three proteins, versus those containing only  $\alpha$  and  $\beta$  is reflected in the change in enthalpy (Fig. III-6B).

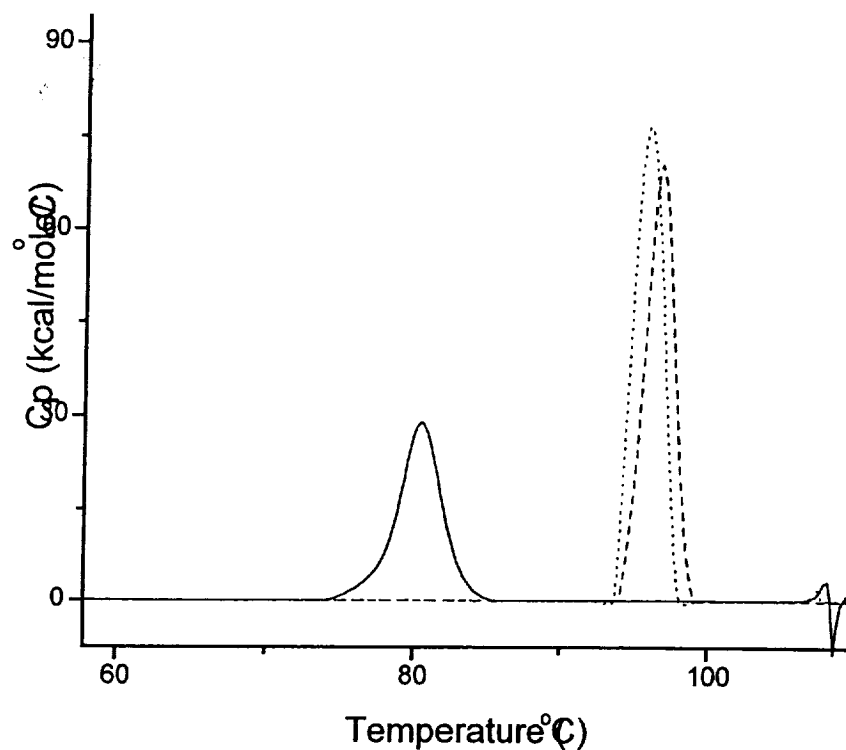


Fig. III-6A. Differential Scanning Calorimetry data showing the thermal denaturation of recombinant forms of the  $\alpha$  (dotted),  $\beta$  (dash) and  $\gamma$  (solid) subunits. The melting temperature,  $T_m$  or thermal midpoint of transition, of the  $\gamma$  subunit is centered at 80.5°C, while the more stable subunits,  $\alpha$  and  $\beta$ , depict  $T_m$  values of 95.7 and 96.7°C respectively.

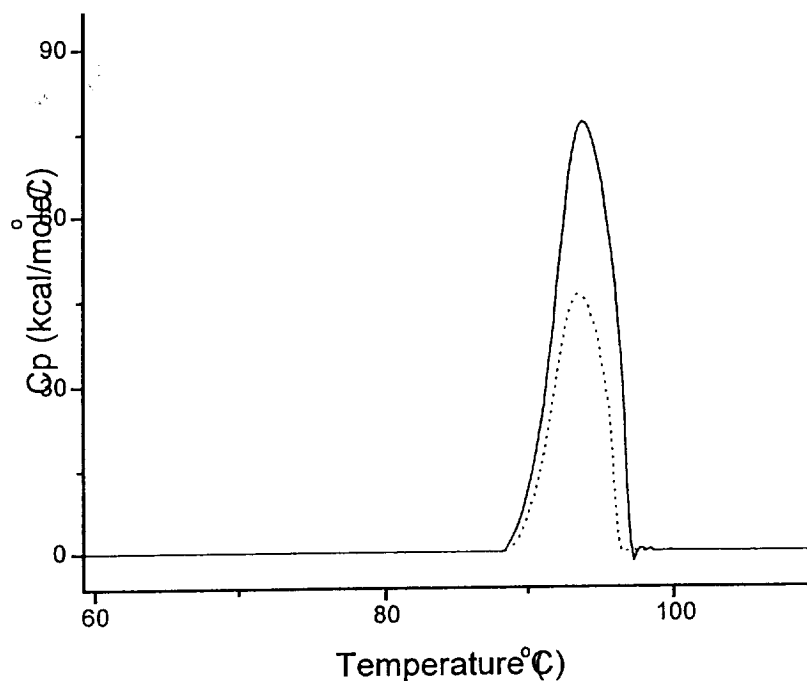


Fig. III-6B. Thermostability of rings formed by the addition of ATP/MgCl<sub>2</sub> to mixtures of recombinant chaperonin subunits and incubation at 60°C for 1 hr. Rings comprised of a 1:1 ratio of  $\alpha$  to  $\beta$  denature at 93.8°C. Rings comprised of a 1:1:0.1 ratio of  $\alpha$  to  $\beta$  to  $\gamma$  denature at 93.5°C. Although the  $T_m$  values are similar, the values of  $\Delta H$  differ significantly.

## DISCUSSION

Contrary to the report by Archibald et. al. (1999), our results suggest that the chaperonin complex in *S. shibatae* is not composed of an equimolar mixture of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits either at low, normal, or heat shock temperatures. Our results indicate that the chaperonin complex, which was believed to be composed of heat shock proteins, contains a subunit that is not heat inducible. Observations both at transcription and translation levels indicate that the  $\gamma$  subunit increased at low temperatures, while both the  $\alpha$  and  $\beta$  subunits increased during heat shock. This suggests the  $\gamma$  subunit may play an important role in the cell at low temperatures, while  $\alpha$  and  $\beta$  are more important at heat shock temperatures. Since the  $\gamma$  mRNA level increases 10-fold by 60 min upon the temperature downshift while  $\alpha$  and  $\beta$  increase 5 to 10-fold in 30 min upon heat shock then decline to the control level in 60 min, the  $\gamma$  may be required for the maintenance state in the cell whereas  $\alpha$  and  $\beta$  for the transition process. The fact that the  $\gamma$  subunit does not form a ring complex by itself suggests it requires interaction with other subunits to be in the ring. That is supported by the observation that the ratio of  $\gamma$  subunit in the ring does not exceed 20% even at a low temperature. To elucidate the function of the  $\gamma$  subunit, electron microscopy is planned to see how the  $\gamma$  subunit affects the higher-order structure of  $\alpha$  and  $\beta$  complexes and further experiments with liposomes are planned to see how  $\gamma$  interacts with  $\alpha$  and  $\beta$  to influence

the stabilization of lipid. In addition, establishing a transformation system for *S. shibatae* would be helpful for investigating the in vivo function of the chaperonin subunits.

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August 26, 2002

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Re: Patent Report for Close-out of Cooperative Agreement NCC 2-1111

Dear Nonnie,

The PI has advised us that no patent was applied for and no reportable inventions resulted out of the work performed under this Cooperative Agreement.

Sincerely,

H.S. Roey  
Manager, Contracts and Procurement, SETI Institute

cc: Robert Padilla NASA Ames Patent Counsel, MS 202A-3  
Hiromi Kagawa, Principal Investigator  
Mr. Ronald Moody, ONR



July 29, 2002

Dept of the Navy  
Office of Naval Research  
RON MOODY  
ADMINISTRATIVE CONTRACTING/GRANTS OFFICER  
1107 N.E. 45<sup>th</sup> st Ste 350  
Seattle WA 98105-4631

Dear Mr. Moody,

No NASA or other Government property is accountable under Grant NCC2-1111.

Please contact me with any questions at 650-960-4526.

Sincerely,

Sue Lehr  
Property Administrator  
SETI Institute